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Significance of the Golgi Apparatus on the Envelopment of Herpes Simplex Virus 1

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Content

1. Abstract

Herpes viruses comprise capsid, tegument and envelope. Capsids are assembled in the nucleus and transported to the nuclear periphery. Nuclear exit, acquisition of tegument and envelope are not well understood. One theory suggests that the envelope acquired by budding of capsids at the inner nuclear membrane fuses with the outer nuclear membrane releasing capsids into the cytoplasm for re-envelopment. Another theory suggests that virions exit the perinuclear space via vesicle formation. The third theory assumes that virions are intraluminally transported from the perinuclear space into Golgi cisternae for packaging. Alternatively, capsids exit the nucleus via impaired nuclear pores and acquire tegument and envelope by budding at Golgi membranes.

Brefeldin A (BFA), which disassembles the Golgi complex, was used to clarify the dual origin of the viral envelope. Addition of BFA resulted in accumulation of virions within the perinuclear space and RER cisternae indicating that budding at the inner nuclear membrane was not affected whereas transportation of virions away from the perinuclear space and RER was suppressed. It is very unlikely that BFA affects selectively the fusion process of the primary envelope with the outer nuclear membrane. Further, if fusion would have been affected the question arises how did capsids gain access to the cytoplasm. The conclusions can thus be drawn that accumulation of virions within the perinuclear space and ER is rather the result of perturbed transportation into Golgi cisternae than of perturbed fusion, and naked capsids more likely gained access to the cytoplasm via exit through impaired nuclear pores than by fusion of the primary envelope with the outer nuclear membrane.

2. Introduction

2.1. Herpes Simplex Virus Type 1

Herpes simplex virus type 1 (HSV-1), the archetype of alphaherpesvirinae, is composed of the core containing the linear double-stranded DNA genome, the icosahedral capsid built of 162 capsomers, the tegument surrounding the capsid, and the envelope consisting of a lipid bilayer with embedded glycoproteins (Roizman, 2001) .

2.1.1. Capsid

The capsid is a well-defined icosahedron which contains and protects the viral genome (Zhou et al., 1999). The icosahedral capsid shell is 16 nm thick, has a diameter of 125 nm (Zhou et al., 1998) and has been described in great detail by cryo-electron microscopy (Newcomb et al., 2000, Zhou et al., 2000) and recently by electron tomography (Grunewald et al., 2003). It houses the DNA, densely coiled in a "liquid crystalline" arrangement (Booy & Moxon, 1991). Capsids have been considered to exist within the nucleus in three forms: B capsids containing scaffolding proteins are the progenitors of C capsids containing viral DNA and empty A capsids, the progenitor of B capsids. Capsids are eccentrically located and occupy about 1/3 of the entire viral volume (Grunewald et al., 2003).

2.1.2. Tegument

Tegument proteins are typically defined as being those structural proteins that are not components of purified capsids or of the envelope. Several of them have been shown to be involved in very early events during infection (Batterson et al.,

1983, Coulter et al., 1993, Fenwick & Everett, 1990, Lemaster & Roizman, 1980, Post et al., 1981), and their presence in the virion ensures their availability at this time. The precise roles of many tegument proteins have not yet been determined and there are several poorly understood aspects of the virus life cycle in which they are likely involved. Among these are packaging and release of the viral genome, transport of the capsid through the nuclear envelope and across the cytoplasm, and formation of the virion envelope (Steven, 1997). An insight into the nature of the tegument came from the identification of a second type of virus particle produced by infected cell, namely the L particles (Szilagyi & Cunningham, 1991). L particles are composed of tegument and envelope but lack of capsids and cores and are consequently non-infectious. Their existence demonstrated that the tegument has inherent structural integrity and that its assembly could take place independently of capsids. The tegument formation can occur in the absence of several of its major component proteins (Zhang & McKnight, 1993) and at least one of the major tegument proteins can increase several fold in abundance (Leslie et al., 1996). These observations suggest that the tegument does not have a unique geometrical organization with every protein occupying a specified position as in the capsid but rather that its constituent proteins interact in variable and possibly semi random ways. It seems that the capsid and tegument will form specific interactions (Zhou et al., 1999).

2.1.3. Envelope and Glycoproteins

The envelope consists of a lipid bilayer. This bilayer membrane is visualized as a continuous smoothly curved surface, about 5 nm thick. The diameter of virions ranged from 170 to 200 nm, averaging 186 nm (Grunewald et al., 2003). An array of glycoproteins protrud from each virion, making the full diameter, on average, about 225 nm. Distribution of the 11 glycoproteins, as for example gB,

gC, gD, has been studied by electron tomography that revealed 595-578 glycoproteins per virion (Grunewald et al., 2003). Four of this glycoproteins are required for virus entry (Avitabile et al., 2004) and they are necessary and sufficient to induce cell-cell fusion when expressed from transgenes (Browne et al., 2001, Pertel et al., 2001, Turner et al., 1998).

2.1.4. Morphology

The shape of intact virions is generally round, although some of them appear to be pleomorphic (Grunewald et al., 2003). The function of viral structures, the targeting and the proper assembly of tegument, the formation of the viral envelope, and the pathway of viral egress from infected cells is highly complex and as yet only partially understood.

2.2. Infection of Cells in Vitro

2.2.1. Cell Entry

To initiate infection, the virus must attach to cell surface receptors by four glycoproteins of the HSV-1 envelope. The cell-cell fusion assay serves as a surrogate for virion-to-cell fusion and infected-cell fusion, even though major differences exist between these systems (Avitabile et al., 2004). Herpes virus are assumed to enter cells via fusion of the viral envelope with the plasma membrane whereby capsid and tegument gain access to the cytoplasm (Fuller & Lee, 1992, Granzow et al., 1997, Lycke et al., 1988, Sodeik et al., 1997) or endocytosis is described (Akula et al., 2003, Avitabile et al., 2004, Bodaghi et al., 1999, Campadelli-Fiume et al., 1988, Granzow et al., 1997, Nicola et al., 2003). Fusion of the herpes virus envelope with the plasma membrane per se has

never been demonstrated. Instead, the envelope was shown to fuse only at small areas with the plasma membrane while the plasma membrane underneath the virion invaginates. The virion then enters into this invagination and finally into the cytoplasm through an opening at the lip of the invagination. The fate of this virions remain unclear (Wild et al., 1998).

2.2.2. Transport to the Nucleus

After cell entry capsids are transported towards the nuclear periphery. DNA is released and enters the nucleus via the nuclear pore complex (Sodeik et al., 1997) for transcription and replication. DNA is then inserted into capsids that has been assembled within the nucleus after import of the capsid proteins (Rixon, 1993).

2.2.3. Egress from the Nucleus

Capsids are transported to the nuclear periphery. Their pathway through the nucleocytoplasmic barrier and the acquisition of tegument and envelope are not fully understood (Homman-Loudiyi et al., 2003). It is well established that capsids bud through the inner nuclear membrane into the perinuclear space, concomitantly acquiring an envelope (Granzow, 2001) and tegument proteins (Wild et al., 2002). The fate within the perinuclear space is controversially discussed. One opinion is that the envelope derived from budding at the inner nuclear membrane fuses with the outer nuclear membrane releasing both tegument and capsid into the cytoplasmic matrix (Browne et al., 1996, Card et al., 1993, Church & Wilson, 1997, Gershon et al., 1994, Gong & Kieff, 1990, Granzow, 2001, Granzow et al., 1997, Klupp et al., 1998, Whealy et al., 1991, Zhu et al., 1995). Capsids are then assumed to be transported to the trans-Golgi

network where they are wrapped by Golgi membranes leading to an enveloped virion within transport vacuoles (Mettenleiter, 2004).

Alternatively, it is speculated that virions escape from the perinuclear space via vesicle formation at the outer nuclear membrane (Campadelli-Fiume et al., 1991, Church & Wilson, 1997, Gershon et al., 1973, Granzow et al., 1997, Radsak et al., 1996, Torrisi et al., 1992). These vesicles then need to pass the Golgi complex in an unknown manner for final maturation of virions.

Contradictory to both the fusion and vesicle formation theory is the fact that fully enveloped virions were found in the perinuclear space and rough endoplasmatic reticulum (RER) (Gilbert et al., 1994, Granzow et al., 1997, Nii, 1992, Radsak et al., 1996, Roller et al., 2000, Schwartz & Roizman, 1969, Whealy et al., 1991) implying that virions transported within the RER system. Connectivity between RER and the Golgi complex (O'Donnell et al., 1988, Wang et al., 2001, Wild et al., 2002) makes it very likely that virions are transported from the perinuclear space via RER into the Golgi complex for packaging into transport vacuoles (Wild et al., 2002). If this theory of intracisternal transport is correct an alternative pathway for capsids entering the cytoplasmic matrix must exist as has been postulated for HSV-1 (Brown et al., 1994).

2.2.4. The Theories of Viral Egress in Detail

2.2.4.1. Theory of De-envelopment and Re-envelopment

The most intensely propagated theory assumes that virions in the perinuclear space are de-enveloped and then re-enveloped at the trans Golgi network. The first budding in herpes virus maturation at the inner nuclear membrane provides the capsid with a primary envelope (Darlington & Moss, 1968, Stackpole, 1969). The envelope of virions within the perinuclear space called primary virions, fuse with the outer nuclear membrane releasing the capsid and tegument proteins such as VP16 into the cytoplasm (La Boissiere et al., 2004, Mossman et al., 2000, Naldinho-Souto et al., 2006). Final envelopment, including the acquisition of more than 15 tegument proteins and more then 10 glycoproteins, occurs by budding of capsids into Golgi-vesicles. These vesicles derived from the Golgi apparatus belong to the trans-Golgi network (Gershon et al., 1994, Granzow, 2001, Jones & Grose, 1988, McMillan & Johnson, 2001, Whealy et al., 1991, Whiteley et al., 1999, Zhu et al., 1995). During re-envelopment, tegumented capsids bud into trans-Golgi vesicles. The orientation of glycoproteins in the vesicle membrane is proposed to be so that the "cytoplasmic tails" may make contact with tegument proteins for driving the final budding process (Mettenleiter, 2002). Mature virions are released after fusion of the vesicular membrane with the plasma membrane.

Arguments for supporting the de-re-envelopment theory are the diverse morphology between virions within the perinuclear space compared to that of extra cellular virions and differences in detection of tegument proteins. Virions in the perinuclear space contain an electron lucent tegument and a dense envelope. Some gene products, e.g. UL31 and UL34 are present in virus particles in the perinuclear space but absent from virions at later stages of assembly (Fuchs et al., 2002b). In contrast, the UL36, UL37, UL49, UL46,

UL47, and UL48 tegument proteins are found only in re-enveloped PRV (Pseudo Rabies Virus) particles and are therefore presumably not recruited into the virion during primary envelopment (Fuchs et al., 2002a, Granzow et al., 2004, Kopp et al., 2002). The PRV US3 protein kinase, on the other hand, is a component of both primary and mature enveloped virions (Granzow et al., 2004). Little is known about the tegument composition of HSV particles at different stages of virion morphogenesis. There are some similarities between HSV-1 and PRV in so far that HSV-1 homologues of UL31 and UL34 are not present in extra cellular virions and an HSV-1 mutant lacking US3 fails to undergo efficient egress from the nucleus into the cytoplasm, suggesting that it is a component of primary virions (Reynolds et al., 2002).

In HSV-1 immunogold electron microscopy was used to determine whether three of the major tegument proteins, VP22, VP16, and VP13/14 (the homologues of the PRV UL49, UL48, and UL47 gene products) are components of primary enveloped virions. Viruses, which have been engineered to express GFP or YFP-tagged fusion proteins of VP16 (La Boissiere et al., 2004), VP22 (Elliott & O'Hare, 1999), or VP13/14 (Donnelly & Elliott, 2001) were used and so each tegument protein could be detected with an antibody to GFP rather than with antibodies specific for each individual protein. VP16 of HSV-1 was demonstrated to be present in virions within the perinuclear space and in extra cellular ones. VP13/14 and VP22, on the other hand, was labelled in extra cellular virions with an incidence of 1.00-1.47 gold particles/virion whereas the average number of gold particles varied between 0.06-0.08 in virions within the perinuclear space. It has to be born in mind that the number of extra cellular virions examined varied between 400 and 800 virions whereas the number of virions within the perinuclear space was only 20 to 40 (Naldinho-Souto et al., 2006).

2.2.4.2. Theory of Vesicle Formation

Another theory assumes that virions exit the perinuclear space by vesicle formation at the outer nuclear membrane (Campadelli-Fiume et al., 1991). Afterwards vesicles bearing virions are transported to the Golgi apparatus for further maturation (Di Lazzaro et al., 1995, Gershon et al., 1994, Lee et al., 1987, Morgan et al., 1954, Poliquin et al., 1985, Torrisi et al., 1992, Ward et al., 1994) and then to the plasma membrane for exocytotic release. The crux of this theory is how vesicles pass the Golgi complex. The vesicular membrane would have to fuse with Golgi membranes to release virus particles into Golgi cisternae which were found to contain virions of HSV-1 (Poliquin et al., 1985), HSV-6 (Torrisi et al., 1999), and BHV-1 (Wild et al., 2002).

2.2.4.3. Theory of two Different Pathways

These theory assumes that capsids exit the nucleus either by budding at the inner nuclear membrane or via impaired nuclear pores (Leuzinger et al., 2005, Wild et al., 2005, Wild et al., 2002). First, nuclear envelopment is suggested to include budding of capsids at the inner nuclear membranes into the perinuclear space whereby tegument and a thick electron dense envelope are acquired. The substance responsible for the dense envelope is speculated to activate intraluminal transportation of virions via RER into Golgi cisternae. Within Golgi cisternae, virions are packaged by fission into transport vacuoles containing one or several virions. Second, for cytoplasmic envelopment, capsids are assumed to gain direct access from the nucleus to the cytoplasm via impaired nuclear pores. Indeed, nuclear pore impairment was demonstrated by electron microscopy (Leuzinger et al., 2005) employing a method for improved retention of cellular material even if they are involved in degradation (Wild et al., 1997). Then, cytoplasmic capsids can bud at the outer nuclear membrane, at

membranes of RER, Golgi cisternae, and large vacuoles, and at banana-shaped membranous structures that were found to continue into Golgi membranes. Envelopes originating by budding at the outer nuclear membrane and RER membrane also acquire a dense substance. Budding at Golgi stacks, designated wrapping, results in single virions within small vacuoles that contain electron-dense substances between envelope and vacuolar membranes.

In contrast to the de-envelopment theory, many investigations clearly demonstrated that "primary" virions are transported from the perinuclear space into the RER cisternae (Gilbert et al., 1994, Granzow et al., 1997, Nii et al., 1968, Radsak et al., 1996, Roller et al., 2000, Schwartz & Roizman, 1969, Whealy et al., 1991) and that "primary" wild-type virions can accumulate within the perinuclear space-RER compartment (Leuzinger et al., 2005, Stannard et al., 1996). Intraluminal accumulation of virions has also been explained due to lack of US3 protein in PRV (Klupp, 2001) or to the absence of gK. GK was, therefore, proposed to be involved in de-envelopment in PRV (Foster & Kousoulas, 1999, Foster et al., 2004) whereas gK does not participate in de-envelopment of HSV-1 (Melancon et al., 2005).

The de-envelopment theory also does not consider that membrane fusion is a fast but well-studied process starting by close apposition of the membrane to allow fusion followed by pore formation (Kanaseki et al., 1997, Kozlovsky et al., 2002, Melikyan & Chernomordik, 1997). Instead, interactions of virions with the outer nuclear membrane were found to have striking features of budding capsids (Wild et al., 2005). This indicates that capsids are able to bud from the cytoplasm into the perinuclear space.

2.3. Effects of Brefeldin A

Brefeldin A (BFA) is a fungal metabolite, which due to its effects on the assembly of membrane coat proteins and on vesicles formation, has provided unique insights into mechanisms of intracellular transport (Klausner et al., 1992). BFA blocks pre-Golgi and intra-Golgi vesicular traffic by inhibiting the formation of transport vesicles. Blockage of vesicular traffic is accompanied by a rapid tubulation of Golgi cisternae, trans-Golgi network, and endosomals compartments (Lippincott-Schwartz et al., 1991). As a consequence the Golgi apparatus disappears and Golgi components are redistributed into the RER (Lippincott-Schwartz et al., 1990, Lippincott-Schwartz et al., 1989). Exploration of Golgi dynamics in living cells (Sciaky et al., 1997) has shown that BFA-induced tubulation occurs during a period of 5-10 min following by a sudden and rapid disassembly of the Golgi apparatus within 15-30 s that leads to enrichment including membrane proteins and lipids in the RER.

Electron microscopy has provided valuable information on BFA-induced ultrastructural changes of the Golgi apparatus. The first transformations were to occur very early after BFA administration (Pavelka & Ellinger, 1993), apparently coinciding with the removal of coat proteins from Golgi membranes (Donaldson et al., 1990, Orci et al., 1991, Robinson & Kreis, 1992). Electron microscopy also confirmed subsequent tubulations and revealed distinct glomerular structures that persist in BFA-treated cells (Hendricks et al., 1992, Hidalgo et al., 1992, Pavelka & Ellinger, 1993). Recent experiments described the Golgi breakdown as not to proceed gradually but occurred in distinct steps (Hess et al., 2000). They found a conspicuous lag between the absence of nonclathrin coats on Golgi membranes after 30 s of BFA treatment and the disassembly of the stacks that did not start until 90 to 120 s. At this time, domains at the *trans* and *cis* faces became separated from the stacks starting tubulation and fragmentation. In addition, they could demonstrate a complete

replacement of the Golgi apparatus by a loose meshwork of straight tubules of different sizes and staining properties after 3-5 min. After 8 min all kinds of Golgi-derived structures had aggregated within huge clusters (Hess et al., 2000).

2.4. Aim of the Study

BFA has been repeatedly used in order to clarify herpes virus envelopment (Chatterjee & Sarkar, 1992, Cheung et al., 1991, Dasgupta & Wilson, 2001, Eggers et al., 1992, Jensen & Norrild, 2002, Koyama & Uchida, 1994, Whealy et al., 1991). It was shown that virus produced by BFA treated cells are not infectious which led to the conclusion that secondary envelopment at Golgi membranes is essential (Koyama & Uchida, 1994, Whealy et al., 1991). If, however, the idea of the dual pathway is correct it will be expected that the proportion of virions originating by budding at the nuclear membrane is infectious provided glycoproteins and other proteins essential for infectivity are inserted into the envelope at this stage. In order to prove this idea, HSV-1 infected cells were treated with BFA after various time periods of inoculation. The obtained data indicate that virions get stuck within the perinuclear space and RER. These virions are shown to be infective suggesting that glycoproteins become part of the viral envelope in the course of budding at nuclear membranes.

3. Material and Methods

3.1. Cells and Viruses

Vero cells (European Collection of Cell Cultures) were grown in Dulbecco's modified minimal essential medium (DMEM; Invitrogen, Basel, Switzerland) supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml) and 10% fetal calf serum (FCS; Omnilab, Mettmenstetten, Switzerland). Wild-type HSV-1 strain F was propagated in Vero cells.

3.2. Infection of Cells

Vero cells were infected with HSV-1 at a multiplicity of infection (MOI) of 5 in DMEM without FCS and kept at 37°C for 1h to allow adsorption prior to incubation at 37°C in DMEM supplemented with 2% FCS.

3.3. Brefeldin A

BFA was added in concentrations of 0.1 µl/ml 2, 4 and 8 h after infection of Vero cells with HSV-1. Infected cells were incubated for 20 hours. Virus yields were determined by plaque titration at 2, 4 and 8 h of infection without BFA, and at 20 h of infection after exposure to brefeldin A.

3.4. Low-Temperature Transmission Electron Microscopy

Cells grown on sapphire disks were frozen in a high-pressure freezing unit (HPM010; BAL-TEC Inc., Balzers, Liechtenstein) as described in detail (Wild

et al., 2002). The samples were then transferred to a freeze-substitution unit (FS 7500; Boeckeler Instruments, Tucson, Arizona) precooled to -88°C for substitution with acetone and subsequent fixation with 0.25% glutaraldehyde and 0.5% osmium tetroxide at temperatures between -30°C and +2°C to achieve good contrast of membranes (Wild et al., 2001), and embedded in Epon at 4°C followed by polymerization at 60°C for 2.5 days. Serial sections of 60 to 90 nm thickness were analyzed in a transmission electron microscope (CM12; Philips, Eindhoven, The Netherlands) equipped with a slow-scan CCD camera (Gatan, Pleasanton, CA) at an acceleration voltage of 100 kV.

3.5. Morphometric Analysis

Images of Golgi complexes were collected from cells 9, 12, 14 and 17 h after infection with HSV-1 at MOI 5 or mock infected cells. Volume density and surface density of the Golgi complex were estimated by morphometric analysis on 30 cells selected at random at a final magnification of 89'500 applying the point counting method (Weibel, 1979). The data were calculated on the basis of the equations: $V_{vg} = P_g/P_{cy}$, and $S_{vg} = I_g/P_{cy}$ x d, whereby V_{vg} is the volume density, S_{vg} the surface density of the Golgi complex, I_g are intersections on Golgi membranes, P_g are points hitting the Golgi complex, and P_{cy} are points hitting the cytoplasm. The data were expressed as μm^3 volume or as μm^2 surface area per 1000 μm^3 cytoplasm.

4. Results

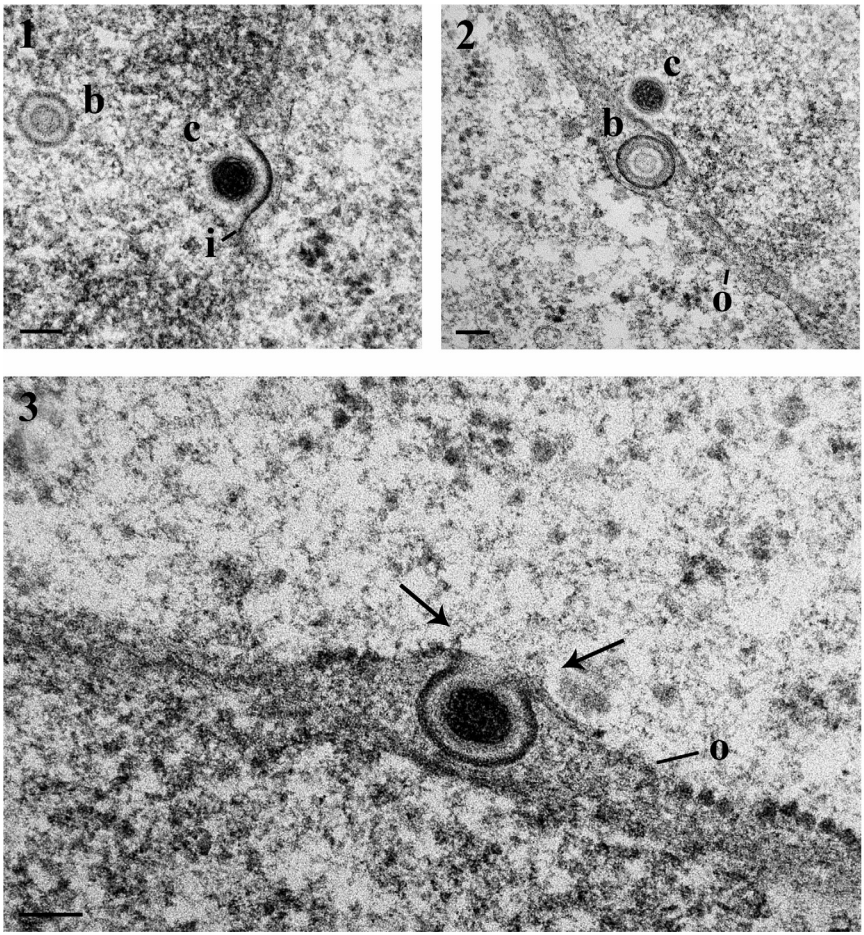
4.1. Envelopment of HSV-1

Vero cells grown on sapphire disk were infected with HSV-1 at MOI 5. The virus production and egress were arrested after incubation at 37°C for 15 to 19 h by immediate freezing. After freeze substitution and embedding serial sections were cut parallel to the smooth surface from which the sapphire disks had been removed. This procedure enabled examinations of virus-cell interactions in an *in situ* situation with a delay of less then 15 seconds between removal of the sapphire disks from the incubation chamber and freezing. All processes of envelopment at the nuclear membrane, RER, and Golgi complex could be readily visualized due to the well-preserved ultrastructure. We could detect basically identical phenotypes to those reported earlier (Leuzinger et al., 2005). Capsids escaping the nucleus via budding at the inner nuclear membrane or via impaired nuclear pores, budding capsids at all cell membranes except the plasma membrane and mitochondrial membranes, virions within RER and Golgi cisternae and within two diverse vacuoles.

4.1.1. Budding at Nuclear Membranes

For nuclear exit via budding, capsids were initially in close apposition to the inner nuclear membrane which was slightly thickened and indented into the perinuclear space. It was remarkable that the thickening always expanded exactly from the side where the membrane indentation started (Fig. 1). The thickening continues to the other side and formed a protrusion in which the capsid was located. At intermediate to late stages, capsids were embedded in tegument and surrounded by a dense envelope which still continued into the inner nuclear membrane. In the perinuclear space we found virions with a dense

envelope detached from the inner nuclear membrane (Fig. 2). Similar phenotype of capsid-membrane interactions were found at the outer nuclear membrane (Fig. 3). Comparing fusion and budding the interactions at the inner and outer nuclear membrane seem to be very likely budding events.



Figures 1-3: Vero cells infected with HSV-1 at 12 h post inoculation. Bars, 100 nm.

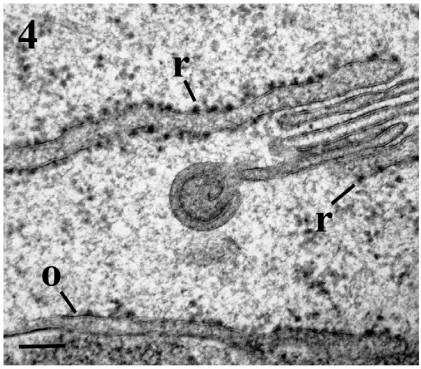
Fig. 1: C-capsid (c) at an early stage of budding at the inner nuclear membrane (i), which is thickened by an electron-dense substance at the site of budding.

Fig. 2: B-capsid (b) within the perinuclear space containing tegument and a dense envelope, probably immediately after fission from the inner or outer nuclear membrane, which is slightly thickened. C-capsid (c) immediately prior to budding at the inner nuclear membrane.

Fig. 3: C-capsid close to completion of budding at the outer nuclear membrane (o). The membrane around the capsid is thickened and turns in a sharp loop (arrows) into the normal outer nuclear membrane.

4.1.2. Perinuclear Space - RER - Golgi Complex

The perinuclear space continued into RER cisternae that contained virions of identical morphology as virions within the perinuclear space indicating that these virions can be transported from the site of origin into RER cisternae. In the quest for destinations of intraluminal virion transportation, we found associations between RER and Golgi membranes (Fig. 4) and virions within laterally dilated Golgi cisternae with strong indications of fission (Fig. 5). The fission of lateral cisternae would lead to vacuoles containing virions. Vacuoles containing one or several virus particles (Fig. 6) consisting of capsid, tegument, and an envelope with distinct spikes were frequently found after incubation for more then 12 h. Additionally we could notice a clear enlargement of the whole Golgi complex during incubation time.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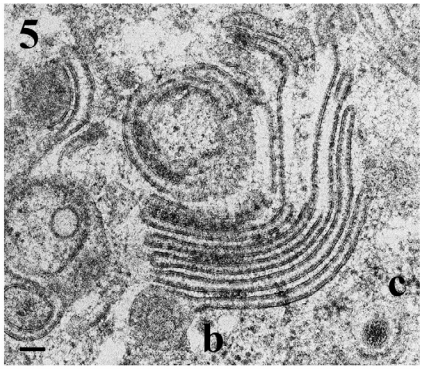
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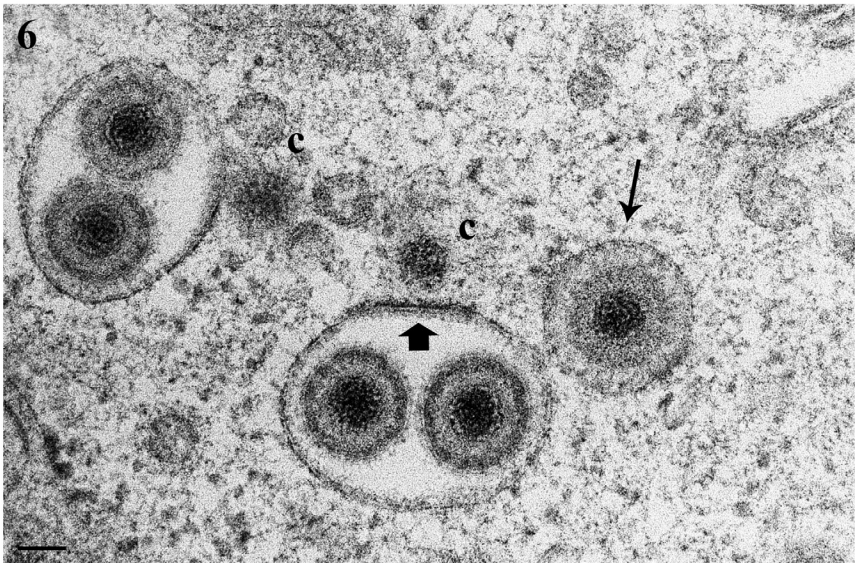

6
c
c

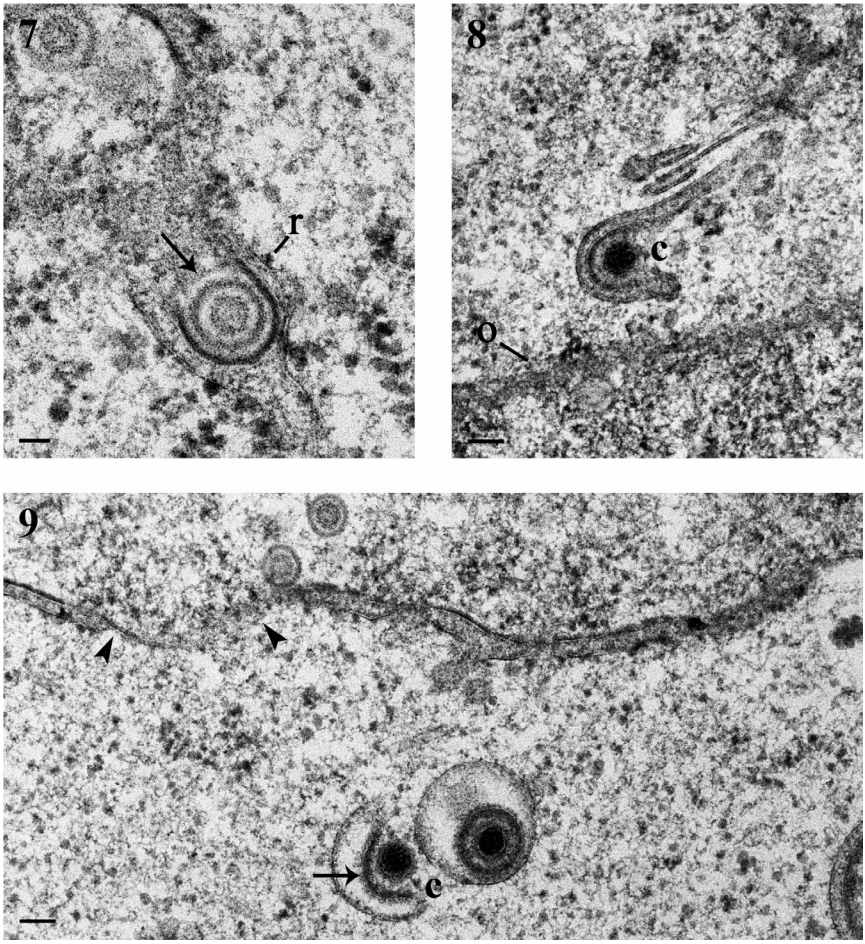
Figures 4-6: Vero cells incubated with HSV-1 for 8 h or 12 h. Bars, 100 nm.

Fig. 4: RER membranes are associated with Golgi membranes. Ribosomes (r) are present at the outer side but are missing at the contact side between RER and Golgi apparatus indicating association.

Fig. 5: Large Golgi complex bearing a virion with a B-capsid (b) at the lateral end of a Golgi cisternae in a late stage of packaging. Budding of C-capsid (c) is shown at a banana-shaped membrane.

Fig. 6: Two vacuoles derived from packaging or cross-sectioned Golgi cisternae, each containing two virions, and a vacuole (thin arrow) with a virion exactly in the center. The space between the envelope and the vacuolar membrane containes a dense substance that might have resulted from wrapping. C-capsids (c) in close apposition to the packaging-derived vacuoles, one of which is in a initial phase of budding (fat arrow).

Capsids were also found to be in close apposition or within indentations at the cytoplasmic side of RER (Fig. 7), Golgi cisternae (Fig. 8) and vacuoles (Fig. 9). The intended membranes were thickened and dense similar as at the inner nuclear membrane and, hence, represent most likely budding events.



Figures 7-9: Vero cells infected with HSV-1 12 h post inoculation. Bars, 100 nm.

Fig. 7: B-capsid at a late phase of budding into RER cisternae from underneath or above the section plane.

Fig. 8: C-capsid in an early phase of wrapping at a slightly thickened membrane of a Golgi complex.

Fig. 9: C-capsid in an early phase of budding into a small vacuole or cross-sectioned Golgi cisternae with a dense deposition at the budding front, and a virion with a vacuole. The area between the arrowheads might represent an impaired nuclear pore.

4.1.3. Nuclear Pores

The question is weather do capsids within the cytoplasm come from if the viral envelope of perinuclear virions does not fuse with the outer nuclear membrane. Close examination of the nuclear surface revealed dilation of nuclear pores through which nuclear material with or without capsids protruded into the cytoplasm (Fig. 10). This finding indicates that capsids may exit the nucleus via impaired nuclear pores to gain direct access to the cytoplasmic matrix.

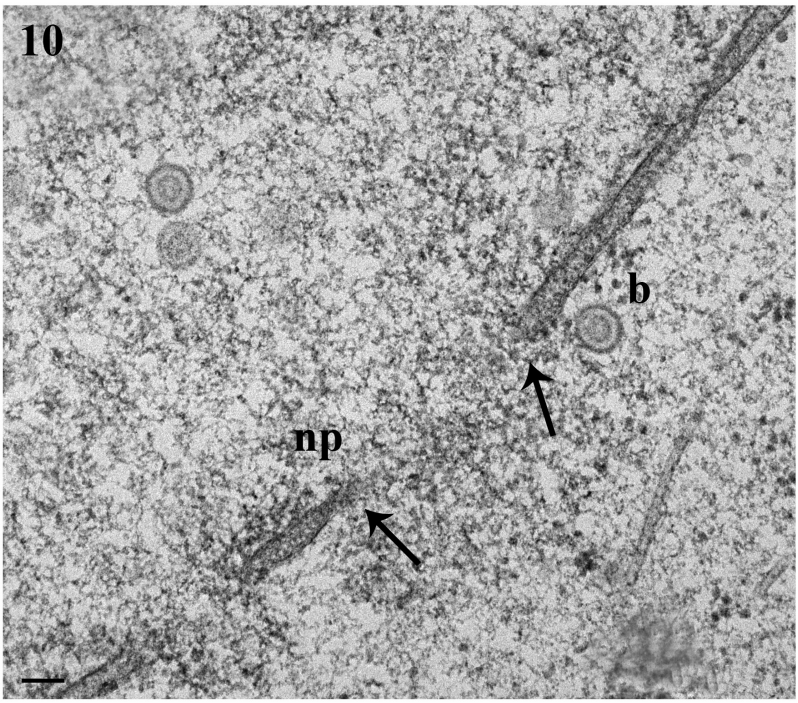


Fig. 10: Impairment of nuclear pores in a Vero cell infected with HSV-1 after 12 h of incubation. Dilated nuclear pore (np) defined by intact nuclear membranes (arrows) and nuclear substance protruding into the cytoplasm. B-capsids (b) have probably escaped through this impaired nuclear pores. Bar, 100 nm.

4.1.4. Distribution of Phenotypes in Envelopment

In an attempt to get an idea about the distribution of the various phenotypes occurring in envelopment capsids at the various stages of envelopment were counted on 20 images taken at random between 8 and 17 h post inoculation. Table I shows that majority of capsids were found on the pathway of nuclear envelopment at 8, 12 and 15 h whereas the number of virus particles following the nuclear pathway was equal to that following cytoplasmic envelopment at 17 h.

Table I: Number of virus particles expressed per 100 µm^2 of cytoplasm in Vero cells incubated for 8, 12, 15 and 17 h after infection with HSV-1 at MOI 5

Phenotypes	8 h	12 h	15 h	17 h
Budding at INM	0	3	0	1
Virions in PNS and RER	16	19	14	3
Virions in P vacuoles or Golgi	9	2	12	4
Total nuclear envelopment	**25**	**23**	**26**	**8**
Naked capsids	2	1	9	3
Budding at ONM	2	2	0	1
Budding at RER	3	0	0	1
Budding at Golgi cisternae	0	1	0	3
Budding at P vacuoles	7	0	3	1
Virions in W vacuoles	0	0	1	0
Total cytoplasmic envelopment	**14**	**9**	**11**	**9**

INM, inner nuclear membrane; ONM, outer nuclear membrane; PNS, perinuclear space; P vacuoles: large vacuoles derived by packaging (Fig. 6); W vacuoles, derived by wrapping: small vacuoles with one virion and an electron-dense substance between envelope and vacuolar membrane (Fig.6).

4.1.5. Size of the Golgi Complex

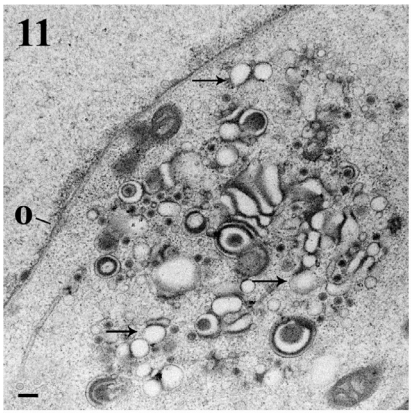
Golgi membranes are used for formation of the viral envelope and also for formation of transport vacuoles containing virions. To fulfil the requirements of large amount of membranes the Golgi complex seems to become enlarged in the course of HSV-1 infection. To ascertain the impression of enlargement of the Golgi complex in HSV-1 infected cells the surface of the Golgi complex was quantified by morphometric analysis (Weibel, 1979) in cells selected at random in a given section. As shown in Table I the surface area was enlarged by 173 % from 9 to 12 h of infection. It was further increased by 198 % at 17 h of infection but decreased to 42 % at 15 h of infection. The volume was also increased by close to 100 % at 12 h of infection. It was however only 56 % larger at 15 h and 17 h of infection. The higher surface area to volume rate at 12 and 17 h of infection indicates that dilation of Golgi cisternae or tortuosity of Golgi membranes were different at 12 and 17 h p.i. compared to 9 and 15 h.

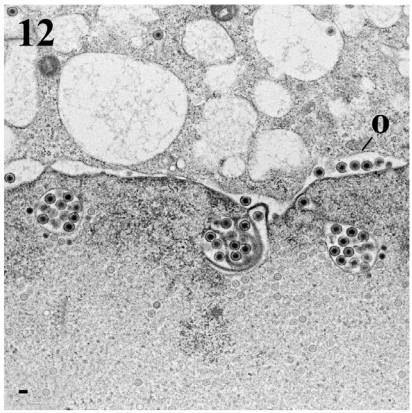
Table II: Volume and surface area of the Golgi complex calculated per 1000 μm^3 cytoplasm in cells infected with HSV-1 at MOI 5

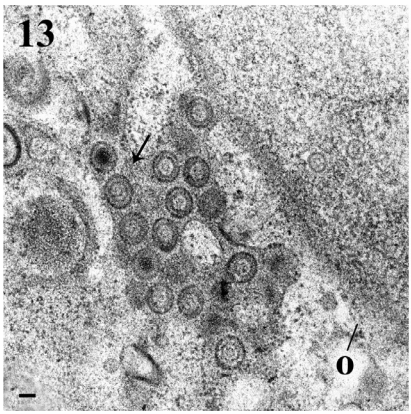
Incubation Time	Surface Area	Volume	Surface/Volume
9 h	253 μm^2	31,8 μm^3	7,9
12 h	691 μm^2	58,4 μm^3	11,8
15 h	436 μm^2	51,9 μm^3	8,4
17 h	756 μm^2	50,0 μm^3	15

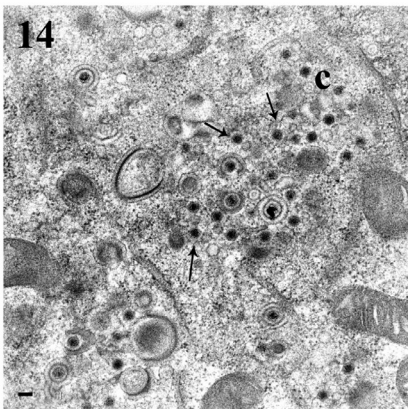
4.1.6. Effects of Brefeldin A on HSV-1 Envelopment

BFA causes disassembly of the Golgi apparatus that rapidly disappears as a morphologically distinct entity. To test whether the theory of the dual origin of the viral envelope is correct we used BFA in concentrations of 1 µl/ml added at 2, 4 or 8 h post inoculation in order to suppress envelopment at the Golgi complex. BFA treatment resulted in disassembly of Golgi cisternae (Fig. 11) and in a dramatic accumulation of enveloped virions between the inner and outer nuclear membrane (Fig. 12) as well as in cisternae of the RER (Fig. 13).









Figures 11-14: Vero cells treated with BFA 5 h after inoculation with HSV-1 and incubated for 15 h (Fig. 11, 13, 14) or 20 h (Fig. 12). Bars, 100 nm.

Fig. 11: Partial disassembly of Golgi cisternae with formation of vacuoles (arrows).

Fig. 12: Accumulation of enveloped virions within the perinuclear space and outer nuclear membrane (o).

Fig. 13: Enveloped virions are restrained within RER cisternae (arrow) due to the disassembly of the Golgi apparatus.

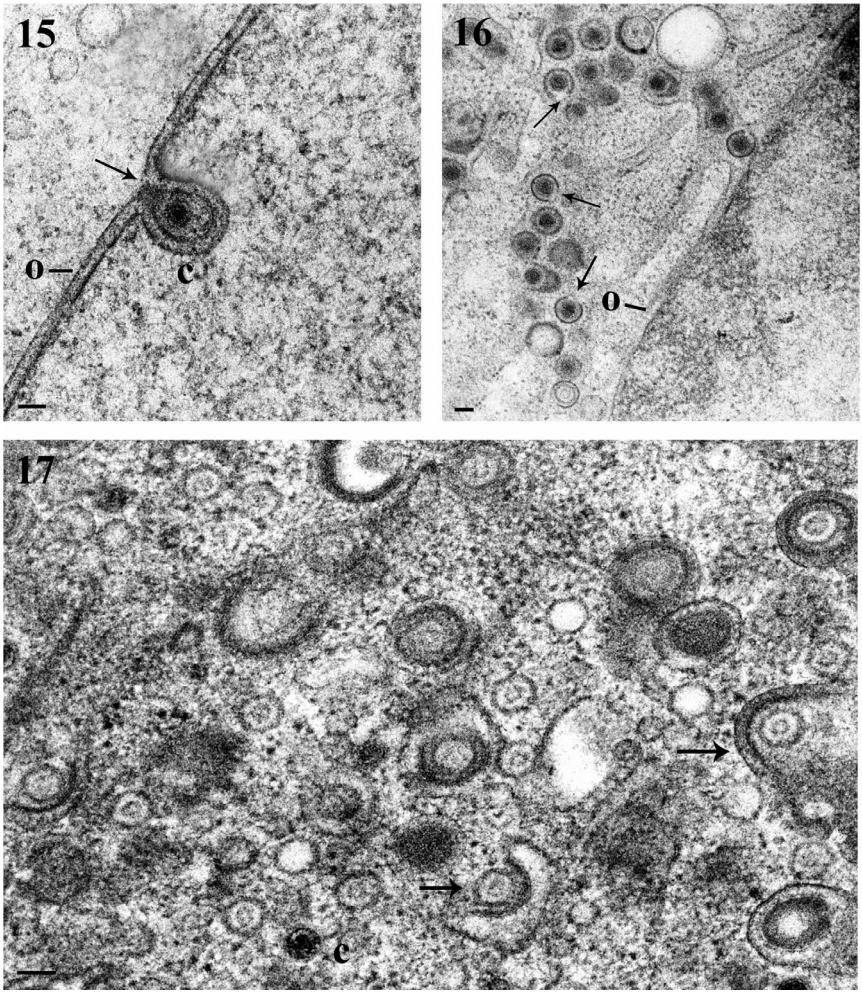
Fig. 14: Accumulation of naked capsids (arrows) within the cytoplasm and some small vacuoles containing enveloped virions indicating that either capsids had bud successfully into disassembled membranes or that Golgi disassembly was not complete.

The mean number of virions within PNS and RER in cells incubated for 20 h in the presence of BFA was 83 when BFA was added 2 h post inoculation. The number of intracisternal virions was 138 and 235, respectively, when BFA was added 4 and 8 h post inoculation. Without BFA treatment the number was only 4. There were no indications for fusion between viral envelopes and outer nuclear membrane. Additionally, many naked capsids accumulated within the cytoplasm (Fig. 14). Capsids approached the outer nuclear membrane (Fig. 15), the RER cisternae (Fig. 16) and some Golgi-derived vacuoles (Fig. 17) from the cytoplasmic side for budding. Most of the remaining Golgi cisternae were vesiculated. Very few virions were found within disassembled Golgi apparatus.

To test whether virions produced by cells treated with BFA are infective, virus were harvested at 20 h of incubation. As shown in Table III, treatment with BFA at 2 or 4 h p.i. resulted in an almost complete loss of infectivity whereas the number of infectious virus was 10^5 when BFA was added 8 h p.i. However, untreated cells incubated for 8 h produced also 1×10^5 infectious viruses.

Table III: Number of virus particles within the perinuclear space and RER cisternae expressed per 100 µm^2 cytoplasm in cells treated with BFA at various time periods after infection with HSV-1 at MOI 5 and incubation for 20 h.

BFA after inoculation	no	2 h	4 h	8 h
Virions within PNS/RER	4	83	138	235
Cytoplasmic capsids	9	19	16	61
IVP: PFU/ml	3×10^7	2×10^2	3×10^2	1×10^5



Figures 15-17: Vero cells treated with BFA 5 h after inoculation with HSV-1 and incubated for 15 h. Bars, 100 nm.

Fig. 15: Late budding event (arrow) of C-capsid (c) at the outer nuclear membrane (o).

Fig. 16: Budding of C-capsids from the cytoplasmic side at RER membranes (arrows).

Fig. 17: Budding of capsids into vacuoles (arrows) presumably developed from disassembled and vesiculated Golgi apparatus.

5. Discussion

Electron microscopic analysis employing high pressure freezing and freeze substitution revealed indications that support the theory of the dual origin of the viral envelope: (i) budding of capsids at all cell membranes except the plasma membrane; (ii) the presence of virus particles consisting of capsid, tegument, and a thick, dense envelope within the perinuclear space and RER cisternae; (iii) virions within Golgi cisternae and (iv) the presence of virions within two diverse vacuoles in the cytoplasm.

In order to clarify the dual origin of the viral envelope BFA was used to abolish envelopment by Golgi membranes. Addition of BFA at 2, 4 and 8 hours p.i. resulted in a dramatic accumulation of virions within the perinuclear space and RER cisternae at 20 h of infection indicating first, that budding at the inner nuclear membrane was not obviously suppressed as reported after short-term treatment with BFA (Dasgupta & Wilson, 2001), and second, that transportation of virions away from the perinuclear space and RER cisternae was suppressed. In addition to accumulation of virions within the perinuclear space and RER cisternae naked capsids accumulated within the cytoplasm. Accumulations of capsids are assumed to be the result of abolished or at least suppressed envelopment by Golgi membranes. The question is where these capsids originate. The fact that they were found adjacent to pockets of enveloped particles in the perinuclear space leds to the interpretation that newly enveloped particles accumulated in the perinuclear space and then fused with the outer nuclear membrane to enter the cytoplasm (Chatterjee & Sarkar, 1992, Cheung et al., 1991, Eggers et al., 1992, Jensen & Norrild, 2002, Whealy et al., 1991). Fusions at the outer nuclear membrane, however, have never been shown. Instead there are clear indications for budding at RER membranes (Fig. 7). It seems very unlikely that disassembly of the Golgi complex affects functional

properties of the outer nuclear membrane whilst functionality of the inner nuclear membrane remains intact as obvious by the high number of virions within the perinuclear space and RER cisternae. Most of these virions have probably derived by budding at the inner nuclear membrane. It seems also very unlikely that virions accumulate in the perinuclear space prior to fusion with the outer nuclear membrane. And, last not least, it is very unlikely that virions with the ability to fuse with the outer nuclear membrane are transported from the perinuclear space into RER cisternae for fusion with RER membranes. The only rational explanation of virus accumulations within the perinuclear space–RER compartment in the absence of the Golgi complex is the breakdown of the intraluminal route from RER cisternae to Golgi cisternae.

For envelopment by budding at the inner nuclear membrane large amounts of membrane constituents are needed that would be inserted into the outer nuclear membrane in the course of de-envelopment. The high number of virions accumulating within the perinuclear space implies that membrane constituents must be supplied either by translocation, e.g. from Golgi membranes, or by de novo synthesis as has been shown to occur in PRV infected cells (Ben-Porat & Kaplan, 1972) and HSV-1 infected cells (Sutter, 2006). If the theory of de-envelopment were correct, membranes used for budding at the inner nuclear membrane would be inserted into the outer nuclear membrane, and consequently, recycle back to the inner nuclear membrane to avoid enlargement of the outer nuclear membrane ad infinitum. In this case de novo synthesis of nuclear membranes would not be needed, at least not for envelopment. If the theory of intraluminal transportation were correct de novo synthesis of inner nuclear membrane constituents would be imperative unless they are supplied from other sources such as the Golgi complex. The Golgi complex is disassembled under the influence of BFA and, hence, membranes could be transported to the nuclear membrane und used for envelopment. If that were true

it would be very difficult to understand why the inner nuclear membrane uses these Golgi derived constituents for envelopment whilst de-envelopment at the outer nuclear membrane becomes impaired. Furthermore, addition of BFA at 2, 4 or 8 h p.i. had a similar effect on virus accumulation though the number of virions was markedly increased when BFA was added at 8 h of infection. This might suggest that the Golgi complex increased during the 8 h of incubation and, consequently, more membrane constituents were available after disassembly for incorporation into nuclear membranes. The size of the Golgi complex, however, was almost identical at 9 h of incubation with HSV-1 compared to mock infected cells (Sutter, 2006). It, however, substantially changed from 9 to 12 hours of infection (Table II). Hence, supply of constituents for the inner nuclear membrane needed for envelopment must have another source than Golgi membranes.

All these considerations support the idea that the release of virions from the perinuclear space is rather intraluminal transportation than by fusion of the viral envelope with the outer nuclear membrane. If capsids within the cytoplasm do not derive by de-envelopment at the outer nuclear membrane an alternative route must exist through which capsids gain access to the cytoplasm. Koyama and Uchida (Koyama & Uchida, 1994) came to the conclusion that HSV-1 envelopment follows two diverse pathways, a BFA-sensitive major pathway and a BFA-insensitive minor pathway. Considering the dual pathway of herpes virus envelopment (Leuzinger et al., 2005) the BFA-insensitive pathway corresponds to the pathway designated nuclear envelopment including budding at the inner nuclear membrane, and intraluminal transportation to the Golgi complex for packaging. The BFA-sensitive pathway corresponds to the pathway designated cytoplasmic envelopment including nuclear exit of capsids via impaired nuclear pores, and budding at Golgi membranes and other cell membranes such as the outer nuclear membrane, membranes of RER and vacuoles.

The function of the Golgi complex in herpes virus morphogenesis is threefold at least. It provides membranes for the viral envelope of those capsids that bud at Golgi membranes. It provides membranes for transport vacuoles, and it is involved in protein synthesis, notably glycosylation of proteins (Alberts, 1994). It is well established that BFA induces alterations in processing and transportation of glycoproteins in mammalian cells (Doms et al., 1989, Kato et al., 1989, Lippincott-Schwartz et al., 1989, Misumi et al., 1986, Perkel et al., 1988, Perkel et al., 1989, Urbani & Simoni, 1990). It was also shown that changes in glycosylation have profound effects on HSV-1 propagation (Campadelli-Fiume et al., 1982, Johnson & Spear, 1982, Olofsson et al., 1988, Peake et al., 1982, Pizer et al., 1980, Serafini-Cessi et al., 1983).

The glycoproteins, gB, gC, gD, gE, gI, gH and gM are embedded in the envelope of HSV-1. Insertion of most of these glycoproteins into the envelope is essential for propagation of infectious virus. Disassembly of the Golgi complex by BFA is expected to abolish glycoprotein processing resulting in the production of non infectious virus. Addition of BFA at 2 and 4 h p.i. resulted in the formation of less virions by budding at the inner nuclear membrane (Table III) and most if not all of these virions were not infective. In contrast, the number of infectious virus produced by cells exposed to BFA at 8 h p.i. was 10^5/ml compared to 10^7/ml in untreated cells. The virus yield was also 10^5/ml in untreated cells incubated for 8 h. Hence, the conclusions can be drawn that these 10^5/ml virus have been produced prior to adding BFA or, alternatively, virions retained in the perinuclear space–RER compartment are infective and contribute – at least in part – to the 10^5/ml infective virus. The first assumption is unlikely because virions do possibly not survive for 12 h after release from cells e.g. as a result of cell death which is apparent by the advanced cytopathic effect. It is thus considered likely that both retained virus in the perinuclear space–RER compartment and virus from early production contribute to the final virus yield.

Table III shows that 2/3 of enveloped virions were in the perinuclear space–RER compartment, and 1/3 in vacuoles derived by packaging. Tegument and envelope of all these virions must have derived by budding at nuclear membranes. Consequently, the 10^5/ml virus harvested at 8 h p.i. have obtained tegument and envelopment via the nuclear pathway exactly as the virions retained in the perinuclear space–RER compartment after treatment with BFA.

If virions from the perinuclear space–RER compartment are infective, glycoproteins must have transported to the nuclear membrane prior to budding of capsids. This transportation of glycoproteins from the Golgi complex to the nuclear membranes must have taken place between 4 and 8 h of incubation. Indeed, immunolabeling revealed that gE (Rychlowski et al., 2001), gK (Hutchinson et al., 1995), gH (Browne et al., 1996), gB (Pertel et al., 1998, Stannard et al., 1996), gC (Oravcova et al., 2000) and gD (Stannard et al., 1996) were present at nuclear membranes. Time course of glycoprotein transportation needs to be studied early in infection. So far gE of bovine herpes virus 1 has been shown to be present at the nuclear rim at 10 h of infection (Briner, 2006). Glycoproteins present at nuclear membranes are assumed to become part of the viral envelope that derives by budding of capsids. Glycoproteins, however, have never been demonstrated on virions in the perinuclear space, probably because they are masked by an electron dense substance of unknown nature (Stannard et al., 1996).

In conclusion, the result of BFA suppressed envelopment support the idea that envelopment of HSV-1 follows two pathways. They also indicate that glycoproteins processed in the Golgi complex are transported to the nuclear membranes where they become part of the viral envelope in the course of budding. Both the theory of the dual pathway and glycoprotein transportation need to be further analysed.

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